guanine was inactivated by treatment with nitrous acid while factor Z_1 was not. On the other hand guanine and factor Z_1 may be two distinct and unrelated compounds each of which is effective on the same cellular system. The relatively high degree of specificity of the known vitamins would militate against this assumption. Furthermore, guanine and factor Z_1 seem to have similar stabilities toward high temperature, acids, alkalies and oxidizing agents. They may, however, be unrelated substances which affect two distinct systems in the *Phycomyces* cell. Answers to these suppositions will probably have to wait the identification of factor Z_1 and its use in pure chemical form.

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RADIOACTIVE CARBON AS AN INDICATOR OF CARBON DIOXIDE UTILIZATION. VIII. THE RÔLE OF CARBON DIOXIDE IN CELLULAR METABOLISM

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I. Introduction.—Previous studies on the metabolism of non-chlorophyllous living systems in the presence of labeled CO_2 ($C^{11}O_2$ or $C^{13}O_2$) have shown that the organisms convert the labeled carbon into organic compounds which may occur as excreted end-products of metabolism, as cellular constituents or both. This phenomenon has been observed with *all* the systems thus far examined[1–12] (*Bacterium coli, Propionibacterium pentosaceum, Methanobacterium Omelianskii, Methanosarcina methanica, Clostridium acidi-urici, Streptococcus faecalis, Acetobacter* species, *Rhizopus nigricans, Rhizopus* species, *Aspergillus niger*, bakers yeast, Lebedev juice, barley roots, plants in the absence of light, rat liver and pigeon liver tissue).

In a few instances the utilization of CO_2 had been clearly demonstrated by methods not involving the use of labeled carbon. (Thus, the utilization of CO_2 by *Methanobacterium Omelianskii*, *Propionibacterium pentosaceum* and *Bacterium coli* had been established by the work of Barker,[13] of Wood and Werkman[14] and of Woods,[15] respectively. Also the reduction of CO_2 to CH_4 by *Methanosarcina methanica* had been predicted on the basis of theoretical considerations.[13] But in the majority of cases where the metabolic activities of the organism or tissue lead to the net production of CO_2, it is not possible to detect a small CO_2 uptake by ordinary chemical methods. Thus it remained for the tracer method to prove the well-nigh universal occurrence of CO_2 utilization.

For a number of years it has also been known that in the complete absence of CO_2, growth and metabolism of diverse living systems are seriously impaired. Under experimental conditions which would insure (1) the initial presence of not more than traces of CO_2, and (2) the prompt removal of additional amounts produced during metabolism, the germination of spores and growth of various microörganisms appeared to be greatly retarded, if not altogether prevented.[16-20] Even such respiratory activities, as methylene blue reduction by "resting cells," were shown to be similarly dependent upon the presence of CO_2.[21] But until now no attempt had been made to interpret these curious results.

The studies with labeled CO_2 have yielded data whose interpretation in terms of general biochemical mechanisms could be attempted. Hence it seemed desirable to investigate in how far such reactions could be of aid in elucidating the effects of CO_2 on growth and metabolism. Admittedly the ideas presented in this paper bear a somewhat speculative character. Nevertheless, they are capable of experimental verification, and may suggest new methods of approach to the many complicated and, at first sight apparently unrelated problems presented by living systems. In this connection may we quote G. N. Lewis[22] ". . . while the sort of vague surmise which is not based upon experimental evidence nor capable of experimental test has no place in our scientific method, rational speculation must always be regarded as the advance guard of experimental science."

II. The Possible Mechanisms of CO_2 Uptake by Living Systems.—One of the most important results of biochemical investigations of recent years has been the demonstration that enzyme-catalyzed processes are, in general, reversible. This implies that a reversal of those reactions in which CO_2 is liberated may be considered as possible mechanisms by which CO_2 is utilized and transformed into organic compounds. A striking example of such a process is furnished by the studies of Woods[15] on the formation of formic acid from CO_2 and H_2 by *B. coli*, in accordance with the equation:

$$HCOOH \rightleftarrows H_2 + CO_2 \qquad (1)$$

More frequently, however, CO_2 production by living organisms is ascribed to an enzymatic decomposition of keto-acids:

$$R\cdot CO\cdot COOH \rightarrow R\cdot CHO + CO_2 \qquad (2)$$

If this type of reaction were reversible, the utilization of CO_2 would then result in the formation of keto-acids[44] whose fundamental rôle and manifold vicissitudes in cellular metabolism have been firmly established.

Experiments reported in a previous communication have shown that the decarboxylation of $CH_3COCOOH$ by yeast carboxylase is indeed reversible.[7] Yet, the reverse reaction appeared to be much too slow to account for more than a very small fraction of the CO_2 uptake observed with living systems.

Investigations on CO_2 assimilation by propionic acid bacteria have suggested a similar, though not identical, method by which this compound is converted into organic substances.[3, 6, 9, 23] The available evidence strongly supports the following mechanism:

$$CO_2 + CH_3\cdot CO\cdot COOH \rightleftarrows COOH\cdot CH_2\cdot CO\cdot COOH^{24} \qquad (3)$$

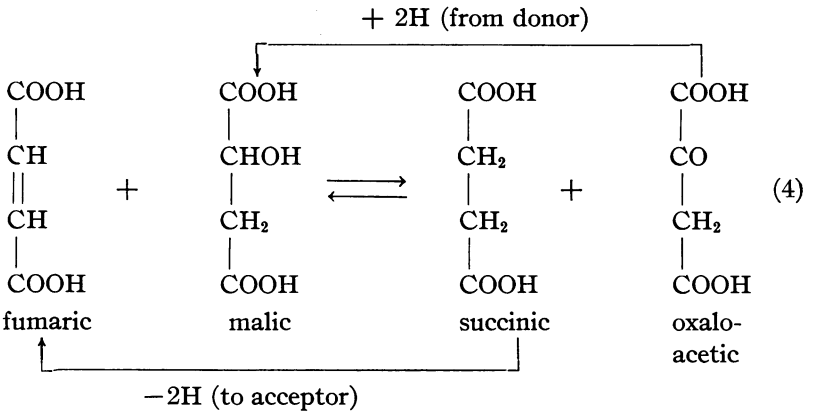
Normally, the oxaloacetic acid is reduced to succinic acid.

Furthermore, the occurrence of this reaction is apparently not limited to the propionic acid bacteria, on the contrary, it seems to be quite widespread. Various types of living systems have been shown to produce succinic and fumaric acids, at least in part, from CO_2. We refer to the work of Krebs and Eggleston with liver tissue,[25] of Smyth with *Staph. aureus*,[26] of Kleinzeller with yeast,[27] of Foster, *et al.*, with molds,[8] of van Niel, Ruben and Thomas with protozoa,[28] of Elsden[29] and of Werkman, *et al.*,[9] with *B. coli*.

It is particularly the general occurrence of a CO_2 fixation mechanism by which oxaloacetic acid and the closely related fumaric and succinic acids are formed which offers an opportunity for a simple interpretation of the above mentioned effects of CO_2 on growth and respiration. For the studies of Szent-Györgyi and co-workers,[30] as well as those of numerous investigators since, have revealed that this group of C_4-acids plays a fundamental rôle in cellular metabolism.

III. The Rôle of the C_4-Dicarboxylic Acids in Cellular Metabolism, and an Attempted Interpretation of the Necessity of CO_2 for Growth and Respiration.— According to our present knowledge in this field, it appears that the biological degradation of a substrate leads to a series of reactions in which electrons or hydrogen atoms are transferred to the ultimate acceptors by way of a rather extensive series of oxido-reduction reactions. In this chain of events, the reversible hydrogenation of oxaloacetate to malate, and of the latter (via fumarate) to succinate, plays the part of a catalytic hydrogen transporting system in various mammalian and avian tissues, as also in

microörganisms. Hence it has been proposed that the C_4-dicarboxylic acids comprise a "catalytic cycle," which can be represented as follows:



This hypothesis implies that the metabolic capacity of a system would depend in part upon the presence of sufficient amounts of the participating C_4-dicarboxylic acids. In growing cells the necessary increase in metabolic capacity must be brought about by a synthesis of one or more of the components of the catalytic cycle, and from the foregoing discussion it appears that such a synthesis can occur from CO_2 and pyruvic acid. If this were the only, or most important, way in which the C_4-dicarboxylic acids *originate*, it is obvious that CO_2 should be an indispensable component of the medium in which growth takes place.

However, these considerations apply rigorously only to conditions under which cell multiplication occurs. Yet, it has been shown by Hes[21] that even in suspensions of "resting cells," CO_2 is necessary for the normal functioning of the respiratory mechanism which results in methylene blue reduction. The explanation of this apparent anomaly becomes clear if one bears in mind the conditions under which Hes' experiments were conducted, in particular the presence of a strong CO_2-absorbing agent. The clue to an understanding of this phenomenon is furnished by the fact that oxaloacetate, apart from playing an important rôle in the Szent-Györgyi catalytic system, can as well undergo a number of other transformations. The most obvious of these is its decarboxylation. And, in the presence of a CO_2-absorbing reagent, this decomposition will proceed to such an extent that the supply of this biocatalyst will gradually disappear. Consequently, it can be asserted that *the decarboxylation of oxaloacetate constitutes a "leak" through which certain essential cell constituents are drained off*. The existence of a simple mechanism by which the components of this catalytic system can be replenished by the interaction of CO_2 and the ubiquitous intermediate product pyruvic acid, thus becomes of great importance as a means of "plugging" this leak.

Nor is the decarboxylation of oxaloacetate the only mode of its elimination from the cycle. From the studies of Knoop,[31] and particularly of Virtanen, *et al.*,[32] one may conclude that the synthesis of dicarboxylic amino acids takes place through a reaction between oxaloacetate or fumarate and ammonia or hydroxylamine. A further possibility for loss of the components of the catalytic cycle is provided by their ready diffusibility. In order to counteract losses by any of these processes, the C_4-dicarboxylic acid supply must therefore be constantly replenished.

Evans and Slotin[10] have recently shown that α-keto glutaric acid, produced by pigeon breast muscle in the presence of C^*O_2, contains radioactive carbon. In view of the work of Krebs and co-workers[33] it is possible to derive the ketoglutarate from oxaloacetate and pyruvate through the proposed citric acid mechanism. Also the recently reported formation of radioactive glycogen in the presence of C^*O_2 (Hastings, *et. al.*[34]) falls in line with the previous considerations. As these authors point out, the most likely interpretation of their experimental results is that the C^*O_2 is used in the synthesis of oxaloacetate. From this latter substance, radioactive pyruvic acid can be formed, which in turn leads to the formation of radioactive carbohydrate.

Whereas these contentions point to the fundamental rôle of a reaction by which oxaloacetic acid is synthesized from CO_2 and pyruvic acid, it does not necessarily follow that this reaction constitutes the only important function of CO_2 in metabolism. It has been emphasized on account of its obvious implications for general metabolism, and also because at the present time it represents one of the possible mechanisms for CO_2 utilization best supported by experimental evidence. Nevertheless, it must be realized that such a formation of oxaloacetate is no more than a special case of the introduction of CO_2 into a molecule by means of reversible decarboxylation.

IV. General Outlook on the Rôle of CO_2 in Biological Syntheses.—That the formation of oxaloacetate is not the only way by which CO_2 enters into cellular metabolism is clear from a consideration of the following well-established facts:

1. The reduction of CO_2 to formic acid by *B. coli* (Woods[15]).
2. The production of CH_4 from CO_2 in the methane fermentation (Barker, *et al.*[4, 13]).
3. The participation of CO_2 in the formation of acetic acid by *Clostridium aceticum* (Wieringa[35]) and by *Cl. acidi-urici* (Barker, *et al.*[5]).
4. The utilization of CO_2 in the formation of urea (Krebs and Henseleit,[36, 37] Evans and Slotin,[11] Rittenberg and Waelsch.[12]
5. The complete synthesis of cell constituents from CO_2 as the only carbon source by all autotrophic organisms.

Closely related to the autotrophic organisms, from the point of view of

synthesis of cellular materials from CO_2, are those microbes which can thrive in the presence of only a single one-carbon compound (CO, HCOOH, CH_3OH, CH_4). In these cases the carbon for all the widely divergent chemical constituents comprising the cell must of necessity be ultimately derived from "one-carbon building stones." If one further remembers that there are numerous cases of facultative autotrophism in which the organism can manufacture its cell constituents either completely from CO_2, or from some simple organic substance (to this class belong all the "hydrogen bacteria," and some "sulfur bacteria") it becomes tempting to suggest the possibility that even in the presence of an organic substrate, syntheses may occur in which CO_2 is one of the reactants. And in view of the fundamental similarity of the most diverse metabolic reactions,[38, 39] it then follows that such a possibility should also be seriously considered for the typically heterotrophic organisms.

The complex manner in which urea appears to be formed from NH_3 and CO_2, indicates that the CO_2 is initially built into a larger organic molecule (ornithine-citrulline-arginine cycle[11, 12, 36, 37]). In essence, this is somewhat similar to the above discussed mechanism for the formation of oxaloacetic acid, although not giving rise to a new carbon-carbon link. Furthermore, it is likely that also in the case of CO_2 reduction to formic acid, the CO_2 is first combined with an organic molecule, conceivably an enzyme, and that it is the reduction of this compound which results in the splitting off of HCOOH. The same reasoning can be applied to the formation of CH_4.

In a sense, therefore, these processes may be viewed as "syntheses of short duration." The formation of acetic acid from CO_2 and H_2 by *Cl. aceticum* furnishes an example in which the carbon atoms from two CO_2 molecules become permanently combined. Also the production of acetic acid by *Cl. acidi-urici* must involve such a synthesis since Barker, *et al.*, have shown that the decomposition of uric acid in the presence of C^*O_2 gives rise to acetic acid in which both carbon atoms are labeled.[5]

These last mentioned syntheses thus form a logical bridge to those processes in which large organic molecules are built up with the aid of CO_2. The step-wise elaboration of carbon compounds from such small elementary units would afford the most flexible mechanism for the synthesis of the endless variety of cellular constituents. Since many microörganisms are capable of effecting these syntheses starting with any one of a large number of simple carbon compounds,† it would seem entirely possible that at least for some of the syntheses CO_2 is used as a building stone. Otherwise it would be necessary to postulate the existence of a large variety of synthetic mechanisms for the elaboration of the same compounds.

So far this discussion has dealt with reactions in which CO_2 plays an important part, but which are independent of light. It has been deduced

from the available evidence that also in photosynthesis the actual CO_2 uptake and reduction can occur in the dark (see especially the striking demonstration by Gaffron that green algae can reduce CO_2 in darkness in the presence of H_2[41]) and that the light only serves to cause a photodecomposition of H_2O,[42] thus providing for a supply of reducing substances. This tends to link the process of photosynthesis directly to all other cases of biological utilization of CO_2.

We have, in the previous section, pointed out that the formation of oxaloacetic acid from CO_2 and pyruvic acid is but a special instance of a reversed decarboxylation reaction. In its most general formulation it can be represented by the equation:

$$RH + CO_2 \rightleftarrows RCOOH \qquad (5)$$

The very generality of this equation renders it perhaps the most adaptable mechanism for CO_2 utilization by living systems. It may well be that future work will demonstrate that the different cases of CO_2 reduction are but variants of this formulation. In this connection the experiments of Ruben, *et al.*, may be mentioned.[1, 43] These investigators allowed green algae to utilize C^*O_2 in the dark and found that the C* assimilated became lodged in a large molecule (molecular weight $\sim$1000), and furthermore showed that a considerable portion of the C* was present in carboxyl groups. Equation (5) was proposed to account for their results.

The general inferences that can be drawn from the preceding considerations lead, we believe, to a unified concept of the rôle played by carbon dioxide in cellular metabolism.

It is a pleasure to express our thanks to Professor H. A. Barker for many discussions and helpful suggestions.

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† E.g., den Dooren de Jong has demonstrated that *Pseudomonas putida* can be grown with any one of some 77 different organic compounds as the sole carbon source, including such diverse molecular species as: saturated and unsaturated fatty acids, hydroxy acids, dibasic and tribasic acids, alcohols, carbohydrates, amines, amino acids, amides, aromatic compounds, etc.[40]

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